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Enhancement of Transfecting Activity of Bacteriophage P22 DNA upon Exonucleolytic Erosion

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The transfecting efficiency of P22 DNA on "rough" strains of *Salmonella typhimurium* or non-restricting mutants of *Escherichia coli* K12 approaches 3×10^{-8} plaques/genome equivalent. It increases 20-fold upon complete erosion of the terminally redundant regions of the DNA molecule with either λ exonuclease or exonuclease III. Eroded DNA molecules form circles and linear oligomers upon annealing. The circular monomers display transfecting activity about ten times higher than that of eroded linear monomers or hydrogen-bonded oligomers. *rec*B *rec*C *sbc*B strains of *E. coli* K12 are transfected with P22 DNA with an efficiency of $1{\cdot}5 \times 10^{-6}$ plaques/genome equivalent. The activity of DNA molecules on these strains is not augmented by erosion. This suggests that the activation by erosion, seen in assays on *rec*$^+$ genotypes, is due to the formation of hydrogen-bonded circular molecules, which more readily escape degradation by the recBC nuclease.

1. Introduction

Transfection of bacterial cells with purified bacteriophage nucleic acids offers an opportunity to study the biological effects of *in vitro* manipulations of these molecules. The transfection is, however, not an efficient process: up to 10^{11} DNA molecules are needed in some cases for a single transfection event (Lawhorne *et al.*, 1973; Trautner & Spatz, 1973). Since a single DNA molecule is often sufficient to bring forth production of phage particles, as judged by the linear response of phage appearance with DNA concentration, and 1 to 5% of molecules are taken up by the cells (our unpublished results), it follows that the overwhelming majority of transfecting molecules undergo degradation.

Comparable degradation does not occur with the DNA injected from infecting phages: their efficiency of infection approaches unity, presumably either because of compartmentalization of the DNA and the host degradative machinery or because of the inhibition of the latter by the phage-coded functions or due to rapid conversion of DNA into resistant structures.

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The efficiency of transfection, i.e. number of infectious centers or plaques produced per genome equivalent, varies widely in different systems; for P22 DNA in $CaCl_2$-treated *Salmonella typhimurium* cells it is close to 5×10^{-8} pge† (Bursztyn *et al.*, 1975), while for λ DNA on similarly treated *Escherichia coli* cells it is about 10^{-4} pge (Mandel & Higa, 1970). Such a difference in efficiency between these two DNAs seemed worth investigating, especially in view of our interest in the biological effects of end-to-end oligomerization of P22 DNA (Sgaramella, 1972).

It is known that one of the intermediates in replication of both λ and P22 DNAs is a circular molecule of unit length (Rhoades & Thomas, 1968; Young & Sinsheimer, 1964), and that λ DNA can form circles *via* its cohesive ends without any further processing, while P22 DNA, having fully base-paired redundant terminal regions, requires exposing complementary single-stranded ends prior to circulation. It is therefore likely that P22 DNA after being injected into the host from the phage has to undergo more steps than λ DNA before being converted to circular molecules. In fact, it has been shown that upon phage infection most of λ DNA is found as covalently closed circles in 5 minutes (Kaiser, 1971), while after the same time no P22 DNA circles can be detected; up to 25 minutes are required for over 50% circularization (Rhoades & Thomas, 1968). It seemed reasonable to us that transfecting P22 DNA molecules will also take longer than λ DNA to circularize; during that time they might be subjected to exonucleolytic degradation by the host enzymes, and therefore display much lower transfecting efficiency. In order to increase the efficiency of transfection of P22 DNA two approaches can be envisaged. One is to modify the DNA structure in such a way as to make it more resistant to cell degradative mechanisms and/or to enable it to enter in less steps the pathways leading to production of phage particles. Erosion‡ *in vitro* of terminally redundant ends in P22 DNA would convert it into a structure with cohesive ends as such similar to that of λ DNA. This should hopefully accelerate *in vivo* conversion of transfecting P22 DNA into covalently closed circles, intermediates in phage DNA replication no longer sensitive to cellular exonucleases. The other approach for increasing transfection efficiency of P22 DNA involves disabling part of the cellular nucleolytic machinery. This paper reports results obtained with both approaches: (1) full erosion of terminally redundant regions of P22 DNA increases about 20-fold its transfectivity; (2) loss of recBC nuclease and exonuclease I due to genetic alterations of the host allows even higher efficiency of transfection.

2. Materials and Methods

(a) *Bacteria*

The bacteria strains used are listed in Table 1.

(b) *Bacteriophages and DNAs*

P22 and λ phages were thermally induced from *S. typhimurium* LT *thy*A57 (P22 *ts*C_2^{29}) and *E. coli* B604 (C_I 857 λ *ind*$^-$) *thy*$^-$, respectively. T7 wild type was grown on *E. coli*

† Abbreviation used: pge, plaques per genome equivalent.

‡ We propose the term "erosion" to signify the stepwise removal of nucleotides from a terminus (either 5′ or 3′) of each strand of a double-helical DNA molecule, leaving the other terminus intact. The resulting DNA molecule will be double-stranded in its central portion and single-stranded at its ends. 1% erosion corresponds to removal of 1% of nucleotides from each end of a DNA molecule. We prefer "erosion" to the more wide-spread "degradation" because this connotes loss of the biological activity.

TABLE 1

List of strains

S. typhimurium	Genotype	LPS character	Reference	Source
TA1659	LT2 ch1-1013 (*Δgal,bio,uvrB*)	Rough	Bursztyn *et al.* (1975)	B. A. D. Stocker
ProC90	LT7 pro AB47	Smooth	Itikawa & Demerec (1968)	P. E. Lobban
SL1027	LT2 *met*A22 *trp*B2H1-*b nml* H2-*e,n,x*(*Fels*$_2$) *fla*A66 *str*A120 *xyl*-404 *met*E551	Smooth	Ornellas & Stocker (1974)	B. A. D. Stocker
SL1654	(Colson CL4419)As SL1027 but hsp LT6 *hsp*S29 *ilv*-452 r^-m^+	Smooth	Ornellas & Stocker (1974)	B. A. D. Stocker
SL1642	(Colson-946)$r_{LT}^-m_{LT}^-$ mutant of proC90	Smooth		B. A. D. Stocker
E. coli				
W5410	F$^-$ *thr*$^-$ *leu*$^-$ *thi*$^-$ *sup*E44 *lac*$^-$ *ton*A21	—		This lab
W5438	r^- m^- *thr*$^-$ *leu*$^-$ *thi*$^-$ *sup*E44 *lac*$^-$ *ton*A21	—		This lab
W5449	r^- m^+ *his*$^-$ *tryp*$^-$ *rec*B *rec*C *sbc*B *leu*$^-$ *arg*$^-$ *pro*$^-$ *thi*$^-$ *ara*$^-$ *lac*$^-$ *gal*$^-$ *mtl*$^-$ *xyl*$^-$ *str*^{r} *tsx*			This lab
W5265	B604 (C$_I$857 λ *ind*$^-$) *thy*$^-$	—		This lab
W3292	coliB *mal*$^-$	—		This lab

B strain W3292. λ and T7 were purified by differential centrifugation, P22 by polyethylene glycol precipitation (Yamamoto *et al.*, 1970), followed by repeated banding in CsCl density gradient. DNAs were extracted by phenol treatment of phage particles, essentially as described by Thomas & Abelson (1966); no more than one nick in 5 molecules could be detected by alkaline sucrose gradient sedimentation.

(c) *Enzymes*

Exonuclease III (460 units/ml, 140 units/mg) and λ exonuclease (0·66 mg/ml) $8{\cdot}8 \times 10^4$ units/mg were gifts of Drs L. L. Bertsch and F. Schachat, respectively.

(d) *Transfection assay*

The procedure of Mandel & Higa (1970) to induce competence has been modified as follows: overnight inocula were diluted 500 times into L (Lennox, 1955) or Penassay (Difco antibiotic medium No. 3) broth and grown at 37°C to $A_{600\text{nm}} = 0{\cdot}6$. The cells were then chilled, pelleted at 12,000 ***g*** in the cold and resuspended in one volume of 0·1 M-$MgCl_2$. They were sedimented, resuspended in 0·5 volume of 0·1 M-$CaCl_2$, kept at 0°C for 20 min and again spun down. The final resuspension was in 0·1 volume of 0·1 M-$CaCl_2$. For the transfection assay DNA (0·01 to 1 μg) in 0·1 ml of SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate pH 7·0) was mixed with 0·2 to 0·4 ml of treated cells. This mixture was incubated for 1 h at 0°C, then for 2 min at 42°C. When *E. coli* or "rough" *Salmonella* strains (Wilkinson *et al.*, 1972) were transfected with P22 DNA, a "smooth" (Naide *et al.*, 1965) indicator (0·2 ml of a stationary phase culture) was supplemented. Top agar (2·5 ml) was added and the cell–DNA mixture was plated.

The procedure described above yielded *E. coli* cells which gave between 400 and 1000 plaques with 1 ng of λ DNA. Transfectivity of λ DNA was not found to be affected by changes in host genotype investigated here, thus, λ DNA was used as a standard transfecting agent for *E. coli* strains.

The number of plaques obtained with P22 DNA varied linearly with its concentration up to about 1 μg DNA/assay (Bursztyn *et al.*, 1975); all the experiments reported here are in the linear range of DNA concentrations.

(e) *Exonucleolytic erosions and annealing of eroded DNA*

The Figure legends describe the conditions of the various erosion reactions. Portions were withdrawn at the indicated times: to stop the erosion, EDTA was added to a final concentration of 20 mM and the samples were heated at 65°C for 3 min and then chilled. They were then either used to monitor the extent of erosion or diluted in 0·1 ml of SSC to determine the transfecting activity of DNA. The extent of erosion was measured as follows (see Sgaramella & Khorana, 1972): 10 to 30 μl of erosion mixture, containing 3 to 10 μg of DNA (spec. act. 2500 to 15,000 cts/min per μg) were spotted on a 2·5 cm × 15 cm DEAE-paper (Whatman DE 81) strip. Descending chromatography in 0·3 M-ammonium formate was used to separate released mononucleotides (R_F about 0·8) from DNA ($R_F = 0$). Paper strips were dried and cut longitudinally in 2-cm long pieces which were counted in a scintillation counter. Uneroded DNA samples contained less than 0·01% of rapidly migrating radioactivity. We could easily assess erosion levels of 0·1%.

Up to 5% erosion with λ exonuclease did not introduce nicks in our DNA samples as measured by alkaline sucrose sedimentation. Comparable erosion with exonuclease III resulted in close to 1 break per DNA strand.

Eroded DNA was annealed essentially as described by Rhoades *et al.* (1968): the samples were adjusted to 2 × SSC, heated at 65°C for 30 min and cooled to room temperature during 15 min: as judged by electron microscopy, less than 30% of molecules from an adequately eroded DNA sample were not annealed.

(f) *Sucrose density gradient sedimentation*

For alkaline gradients 5% sucrose was prepared in 0·2 M-NaOH, 0·8 M-NaCl, 1 mM-EDTA; 20% sucrose in 0·8 M-NaOH, 0·2 M-NaCl, 1 mM-EDTA. For neutral gradients 5% and 20% solutions were prepared in 1 M-NaCl, 20 mM-Tris·HCl, 1 mM-EDTA (pH 7·6).

DNA samples in 0·2 ml of SSC were layered on the top of preformed linear gradients and sedimented in the SW 50·1 rotor using a Beckman ultracentrifuge L2-65 B, at 5°C and 49,000 revs/min for the indicated times. Drops were collected from the pierced bottom of the tubes in vials or directly on glass-fiber filters and analyzed.

(g) *Electron microscopy*

Spreading the DNA samples was performed in aqueous medium according to Inman & Schnös (1970). A Siemens Elmiskop 1A was used to analyze DNA samples. Single-stranded termini at the ends of molecules eroded at least 1% originated collapsed structures with bush-like appearance, which were used to evaluate the fraction of molecules which had been actually eroded.

3. Results

(a) *Increase of transfecting activity of P22 DNA upon erosion*

(i) *Exonuclease III*

Optimal conditions for the action of exonuclease III (Richardson *et al.*, 1964) resulted in an erosion which too quickly went beyond the terminal repetitions of P22 DNA. Therefore, the particular variations of the relevant parameters (pH, temperature and time) described in the Figures were adopted as the most convenient for the present investigation. Accordingly, the top panel of Figure 1 illustrates the erosion of P22 DNA with exonuclease III; no lag is apparent, the release of mononucleotides

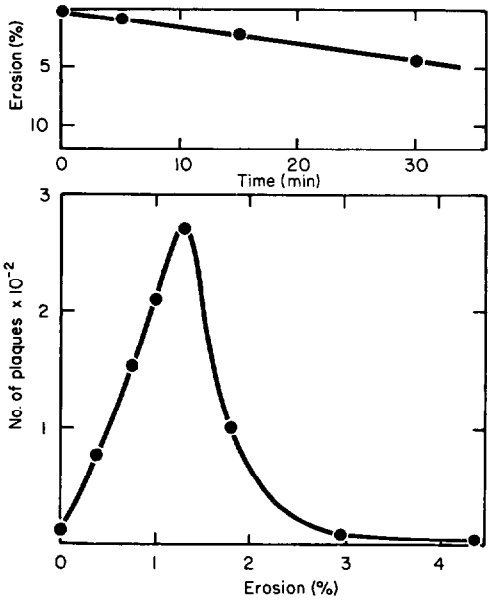

FIG. 1. Enhancement of transfecting activity of P22 DNA by erosion with exonuclease III. The erosion mixture (110 μl) contained 50 mM-Tris·HCl (pH 8·0), 5 mM-$MgCl_2$, 8 mM-2-mercaptoethanol, 30 μg P22 [^{3}H]DNA (spec. act. 2300 cts/min per μg) and 920 units of enzyme. The incubation was at 30°C. Portions were withdrawn at the indicated times and the reaction was stopped as described; the extent of erosion was measured by chromatography on DE-paper and the transfection was performed on the *S. typhimurium* strain TA1659 at a DNA concn of 0·01 μg/assay (see Materials and Methods).

is linear with time at a rate of approximately 75 nucleotides per strand per minute. If allowed to proceed, the digestion produces up to 50% mononucleotides.

The effect of erosion on the transfectivity is shown in Figure 1, bottom panel. Maximal activation is observed when about 1% of nucleotides have been eroded; at this level the efficiency is close to 10^{-6} pge, or about 20 times higher than that observed with uneroded DNA (5×10^{-8} pge). This activation does not appear to be due to the presence of nucleotides released by erosion since: (a) adding 1% of equimolar mixture of the four 5′-P-deoxynucleotides to uneroded DNA does not increase its activity; (b) removal of nucleotides by dialysis from eroded samples does not affect it either. Similarly, the presence of the enzyme seems not to be the cause of activation: (a) 100 times less enzyme brings forth the same activation provided that the same level of erosion is reached by using higher temperature and longer digestion time (this experiment is feasible as exonuclease III is not a processive enzyme (Richardson *et al.*, 1964)); (b) heating to 65°C for 3 minutes, which inactivates the enzyme, does not interfere with the transfectivity of DNA.

(ii) *λ exonuclease*

λ exonuclease, as opposed to exonuclease III, acts processively from the 5′-termini (Little, 1967; Cassuto *et al.*, 1971). Therefore, conditions were established to ensure the saturation of P22 DNA. Figure 2 shows that the substrate is saturated at an

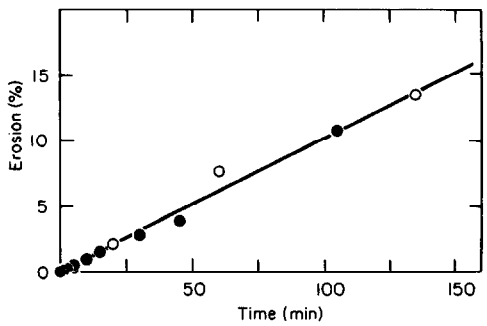

FIG. 2. Saturation of P22 DNA with λ exonuclease.
A reaction mixture containing in 150 μl 66 mM-K-glycinate (pH 9·4), 3 mM-$MgCl_2$ and 45 μg of P22 [^{3}H]DNA was divided into 2 portions: to one, λ exonuclease was added at 16 μg/ml (○), to the other at 6·6 μg/ml (●). The incubations were performed at 20°C.

enzyme:DNA molar ratio of 8, in agreement with published data (Little, 1967). The rate of erosion is close to 45 nucleotides per strand per minute at 20°C and optimal pH. At pH 7 it drops to about 13 nucleotides. This rate appears to be four to five times lower than the one found with T7 DNA (Fig. 3(a)), a result observed also with exonuclease III. The reason for such a difference is not clear: experiments in which the T7 and P22 DNAs were mixed and then eroded with excess of λ exonuclease confirmed the lower susceptibility of P22 DNA under various conditions (Fig. 3(b) and (c)), thus ruling out the contamination of P22 DNA by a diffusible inhibitor. Therefore, the effect might be a consequence of either an association of non-diffusible substances with phenol-extracted P22 DNA or of some unknown sequence differences between the two DNAs.

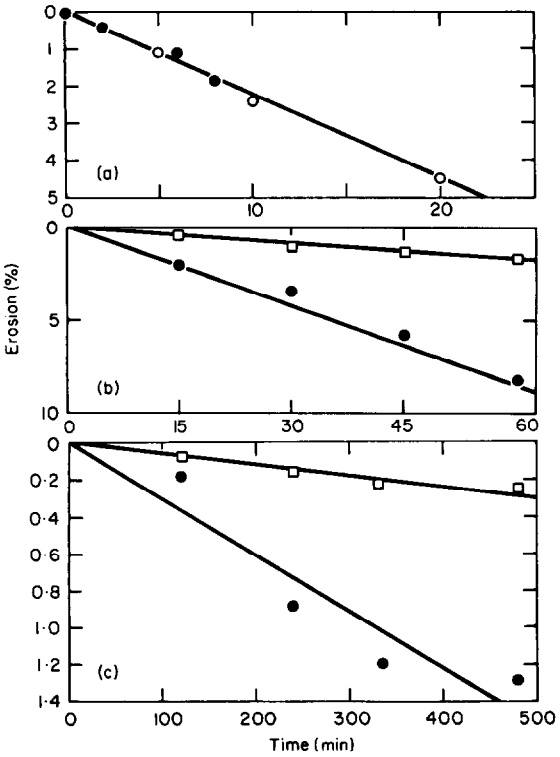

FIG. 3. Erosion of T7 and P22 DNAs with λ exonuclease. (a) 100 μl reaction mixture containing 60 mM-K-TES (pH 7), 4 mM-$MgCl_2$, 32 μg of T7 [^{14}C]DNA (spec. act. 5000 cts/min per μg) and 1·3 μg of λ exonuclease was incubated at 20°C. (b) A 100-μl reaction mixture containing 16 μg of T7 [^{14}C]DNA, 16 μg of P22 [^{3}H]DNA and the components described in (a) were incubated also at 20°C. (c) A 100-μl reaction mixture containing 60 mM-K-glycinate (pH 9·4), 4 mM-$MgCl_2$ 16 μg of each of P22 and T7 DNAs and 1·3 μg of λ exonuclease was incubated at 0°C.

The erosion was determined as described in Materials and Methods: open and closed circles refer to different experiments with T7 DNA, squares to P22 DNA.

The variation of the transfecting activity of P22 DNA with λ exonuclease erosion is shown in Figure 4; as in the case of exonuclease III, the maximum enhancement, close to 20 times, is reached when about 1% of the DNA has been eroded. The processive action of λ exonuclease enabled the following control experiment. A limiting amount of enzyme, sufficient to attack only 2 to 3% of P22 DNA molecules, was allowed to act long enough to release slightly more than 1% of nucleotides. The transfecting efficiency of DNA remained essentially unchanged (Fig. 5, left part). When, after 30 hours, the enzyme was made saturating, the rate of erosion became 40 times higher, and the transfecting activity increased abruptly about tenfold (Fig. 5, right part; note the different time scale). These results indicate that in order to enhance transfectivity of the P22 DNA it is not enough to release by erosion 1% of mononucleotides, by digesting fully a small proportion of the molecules; rather it is necessary to erode partially each DNA molecule.

(b) *Transfecting activity of eroded, annealed P22 DNA*

P22 DNA samples eroded with λ exonuclease to different levels were annealed and analyzed by electron microscopy (Fig. 6). Hydrogen-bonded structures (circles and

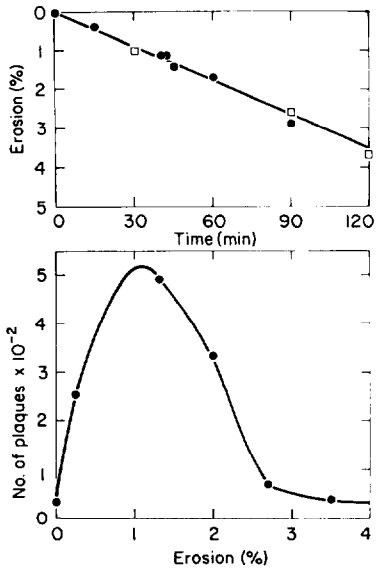

FIG. 4. Erosion of P22 DNA by λ exonuclease and enhancement of transfecting activity.

A reaction mixture (300 μl) containing 60 mM-K-TES (pH 7·0), 4 mM-$MgCl_2$, 4 μg of λ exonuclease and 90 μg of P22 [^{3}H]DNA, was incubated at 20°C. Portions of 50 μl were withdrawn at the various times indicated and the extent of erosion was determined on 10-μl portions. Squares (■) and circles (○) are data from 2 independent experiments. For the transfection assay, 1-μl portions were used.

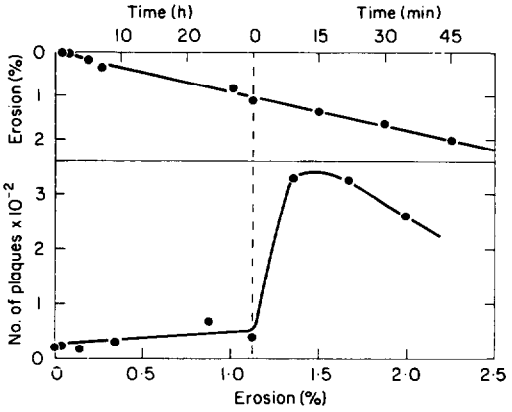

FIG. 5. Effect of λ exonuclease:DNA ratio on transfecting activity.

A mixture of 112 μl containing 60 mM-K-TES (pH 7·0), 3 mM-$MgCl_2$, 32 μg of P22 [^{3}H]DNA and 0·132 μg of λ exonuclease, was incubated at 20°C. Portions of 10 μl were withdrawn at the indicated times and diluted into 100 μl of SSC. After 30 h, 0·66 μg of fresh enzyme were added to the remaining mixture, and the erosion allowed to proceed for 45 min with samples taken at the indicated points and processed as above. The extent of erosion was measured as described on 30 μl of each sample (top half). The remaining part (80 μl) was used for transfection.

The reaction performed with limiting enzyme is presented in the left half, the one with excess of enzyme in the right one.

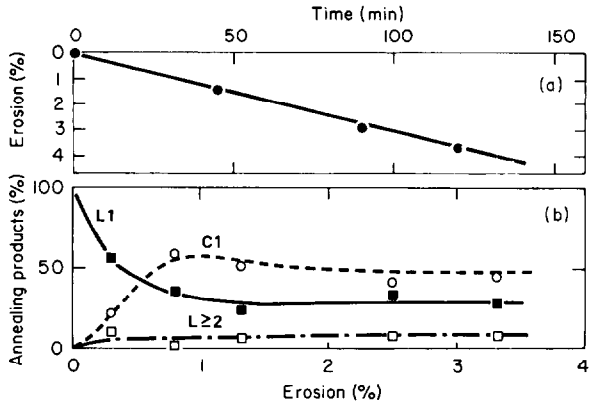

FIG. 6. Frequency distribution of annealing products as a function of concentration and extent of erosion.

The DNA was eroded with λ exonuclease to different levels (a). It was annealed at 5 μg/ml and analyzed by electron microscopy as described in Materials and Methods (b). L1, C1 and L ≥ 2 refer to linear molecules of the size of intact P22 DNA, to circular molecules of the same size and to linear molecules an integral multiple higher, respectively.

linear oligomers) could be seen; their amounts reach a plateau at about 1% of erosion. Maximal activation of DNA transfectivity occurs at the same erosion level (Fig. 4); this suggests that in order to increase the transfecting efficiency, it is necessary to convert molecules to an annealable form by eroding the full length of terminally redundant regions.

The question whether any particular species resulting from annealing would display an enhanced transfecting activity was approached as follows. A sample of DNA was eroded with λ exonuclease to 1·8%; activation achieved was about eightfold

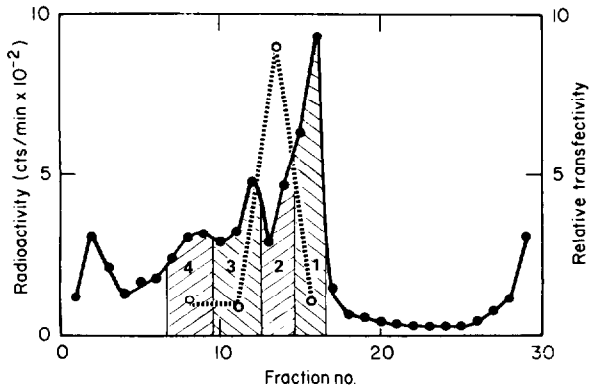

FIG. 7. Sedimentation and transfecting activity of λ exonuclease-eroded, annealed P22 DNA.

P22 DNA was eroded with λ exonuclease in a 100-μl mixture containing 280 μg DNA, 60 mM-K-glycinate (pH 9·4), 4 mM-$MgCl_2$ and 2·1 μg of the enzyme for 20 min at 20°C. The erosion level was 1·8%. DNA was diluted to 10 μg/ml and annealed as described in Materials and Methods. 0·2-ml portions were sedimented in a SW 50.1 rotor for 2 h. Fractions were collected from the bottom of the tube, 10 μl were used for counting and the rest pooled as indicated (hatched areas), dialyzed against SSC and used for electron microscopy (see Table 2) and transfection assays. Radioactivity profile is shown in solid line, relative transfectivity in broken line.

TABLE 2

Transfecting activity and frequency of annealed P22 DNA species fractionated by sedimentation†

Pool no.	Relative sedimentation velocity	Frequency (%) L1‡	C1‡	L ≥ 2‡	C ≥ 2‡	Others§	Transfecting efficiency (pge × 10^7)	Relative transfectivity
1	1	83	13	4	0	0	2·2	1
2	1·2	16	59	24	0	2	20	9·1
3	1·4	12	2	86	0	2	2·0	0·9
4	1·6	15	3	73	9	1	2·2	1·0

† Sedimentation conditions are those described in the legend to Fig. 8. Frequencies shown are contributions to the total mass of DNA. Close to 100 molecules were scored from each pool.

‡ See Table 3 and legend to Fig. 6.

§ Mainly "sigma" and "theta"-like molecules.

($3{\cdot}8 \times 10^{-7}$ pge). Eroded DNA was annealed as described in the legend to Figure 6; the change in transfecting activity was not significant ($2{\cdot}5 \times 10^{-7}$ pge). The annealed sample was centrifuged in a sucrose gradient; fractions were pooled as shown in Figure 7. The pools were analyzed by electron microscopy, and their transfection efficiencies were determined (Table 2). Pool 2, containing predominantly monomeric circles, displays a transfecting activity close to ten times higher than that of pools 1, 3 and 4, which contain predominantly monomeric, dimeric and trimeric linears, respectively. Nevertheless, the transfecting activity of the latter pools is above that of uneroded DNA.

(c) *Circular permutation of P22 DNA*

Rhoades *et al.* (1968) have reported P22 DNA to be extensively circularly permuted, the number of permutations possibly equaling the number of base-pairs in the genome. More recently, Tye *et al.* (1974) have found the permutation to be restricted to not more than 20% of the genome. Our experimental approach can furnish some indications on this problem.

Figure 6 shows that the erosion of P22 DNA leads to the formation of annealable molecules. At an erosion level close to 1%, about 70% of the linear monomers are annealed into either circular monomers or to a lower degree into linear oligomers; the relative amounts of these structures are not affected by further erosion up to at least 3·4%. Electron microscopy analysis of samples eroded to that level, but not annealed, revealed that over 90% of molecules end in bush-like structures, typical of single strands. This suggests that the erosion has proceeded at both ends of almost all DNA molecules, with an asynchrony below 1% of the genome (see Discussion).

Additional evidence of the presence of oligomeric molecules was obtained by zone sedimentation of eroded and annealed P22 DNA (Fig. 8). Intact annealed molecules sedimented as a unique class (Fig. 8(a)), identical to non-annealed DNA (not shown), whereas progressively eroded, annealed samples exhibited increasing proportions of fast sedimenting structures (Fig. 8(b) to (d)) which were identified by electron microscopy as monomeric circles and oligomers of various sizes. The apparent higher

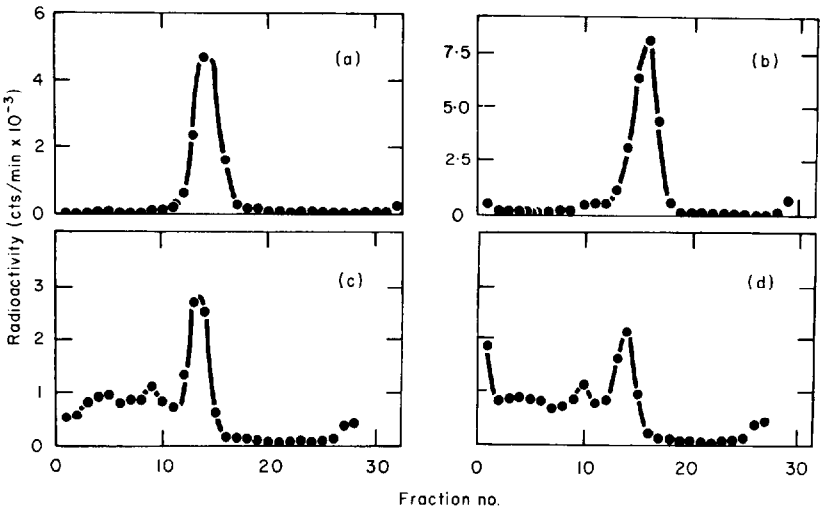

FIG. 8. Sedimentation analysis of eroded, annealed P22 DNA.
A series of variously eroded DNA molecules was prepared as usual ((a) 0%; (b) 0·2%; (c) 1·2%; (d) 2·0%). Annealing was performed at a DNA concentration of 5 μg/ml as described in Materials and Methods. Sedimentation (see Materials and Methods) was for 2 h. Top of the gradients is at the right.

level of oligomers detected by centrifugation as compared to electron microscopy can be explained by the facts that (a) the former method scores the mass, the latter the number of molecules; (b) longer molecules, being intrinsically more difficult to visualize, are underestimated by the electron microscopy analysis.

The formation of oligomers, in addition to circular monomers, is unexpected at the DNA concentration used in our experiment (5 μg/ml) if the circular permutation of the genome is extensive (Rhoades *et al.*, 1968), as the concentration of ends that embody a complementary sequence of sufficient length to form a stable duplex between different molecules would be too low for intermolecular annealing to occur at a measurable rate (Davidson & Szybalski, 1971). An evaluation of the extent of the circular permutation of a genome can be obtained by estimating the concentration of such inter-molecularly complementary sequences. To that purpose, annealing

TABLE 3

Frequency distribution of products formed upon annealing eroded P22 or T7 DNA†

DNA	Erosion (%)	Frequency (%)			
		Monomeric linears	Oligomeric linears	Monomeric circles	Oligomeric circles
P22	1·2	29	8	60	3
T7	0·3	31	11	53	5

† The 2 DNAs were eroded with λ exonuclease and annealed essentially as described in the legend to Fig. 6. For each DNA about 200 molecules were scored by electron microscopy.

properties of eroded P22 DNA were compared to those of eroded T7 DNA. The latter was chosen as it is known to be a non-permuted genome; it is also of a similar size (Studier, 1972) and the length of its terminal repetition (Ritchie *et al.*, 1967) is of the same order of magnitude as in P22 DNA. Table 3 summarizes the result of this comparison, based on the electron microscopy analysis. The two distributions of annealing products were found to be remarkably similar. Thus it appears that the concentration of inter-molecularly complementary ends is similar in the two samples at the same DNA concentration; it can therefore be argued that the P22 genome is not extensively permuted, a finding in agreement with the results reported by other investigators (Tye *et al.*, 1974). In fact, a computer simulation of annealing, using the Jacobson & Stockmayer (1950) formula argues strongly against the number of circular permutations being higher than ten, giving the best fit to the experimental data for a value as low as two (not shown).

(d) *Release of phage from transfected cells*

In order to study the effect of erosion of P22 DNA on the time course and extent of production of phage particles, transfected *S. typhimurium* cells were incubated at 37°C in L-broth and the number of plaque-forming units determined as a function of time. Differently eroded DNAs led to different levels of plaque-forming units (Fig. 9); their initial ratios did not change with time of incubation. This indicates

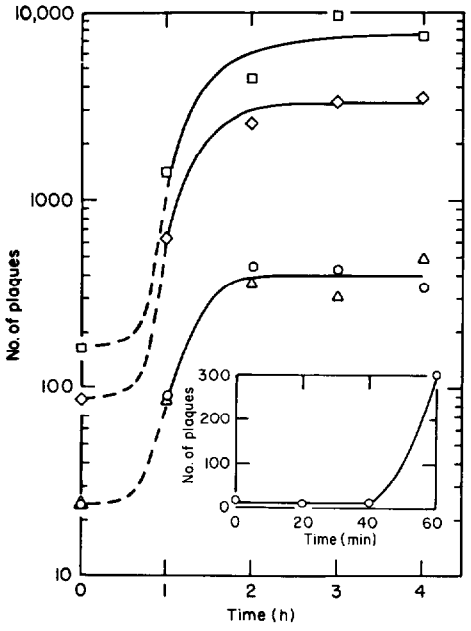

FIG. 9. Production of phage particles upon transfection of $CaCl_2$-treated *S. typhimurium* cells with eroded P22 DNA.

Induction of competence, erosion of DNA and transfection of *S. typhimurium* strain TA1659 were as described in Materials and Methods. Transfected cells were collected by centrifugation, resuspended in 10 vols L-broth and incubated at 37°C. Portions were withdrawn at the indicated times and the number of phage particles determined as described in Materials and Methods. Circles, squares, lozenges and triangles correspond to 0%, 0·8%, 1·5% and 3·2% of erosion, respectively. The inset shows a separate experiment with uneroded DNA; no increase of number of plaques is seen up to 40 min of incubation.

that exposure of single-stranded ends in DNA molecules, although greatly increasing transfecting efficiency, has no effect on either the rate of production of phage particles from transfected cells or on the phage burst size.

(e) *Transfection of* E. coli *with P22 DNA*

Phage P22 does not infect *E. coli* cells but its DNA can transfect *E. coli* spheroplasts (Benzinger & Kleber, 1971). We have studied the transfection of $CaCl_2$-treated *E. coli* cells with P22 DNA carrying *S. typhimurium* S and L modifications (Colson & Van Pel, 1974). In order to detect the formation of phage, the smooth *S. typhimurium* indicator cells (see Materials and Methods), lacking both the S_a and LT restriction systems (Colson & Van Pel, 1974), were added before plating to the transfection mixture.

A number of restriction proficient *E. coli* K12 strains could be transfected with P22 albeit at a very low efficiency: typically a C600 strain exhibited a transfectability of 5×10^{-11} pge. On the other hand, an isogenic restrictionless C600 strain was transfected at an efficiency of 2×10^{-8} pge, which is close to that observed on *S. typhimurium* cells. The competence of the two *E. coli* isogenic strains was normalized as described in Materials and Methods. Transfecting P22 DNA therefore appears to be strongly restricted by the *E. coli* K host specificity system in $CaCl_2$-treated cells, much more so than in spheroplasts (Benzinger & Kleber, 1971).

Since transfecting P22 DNA is a linear molecule, it can be attacked by various exonucleases of the host. It should therefore be expected that *E. coli* strains lacking some of these activities will be transfected at higher efficiencies than their wild-type counterparts. Indeed, a strain deficient in recBC nuclease and exonuclease I (in addition to restriction endonuclease) was transfected at a 100-fold higher efficiency

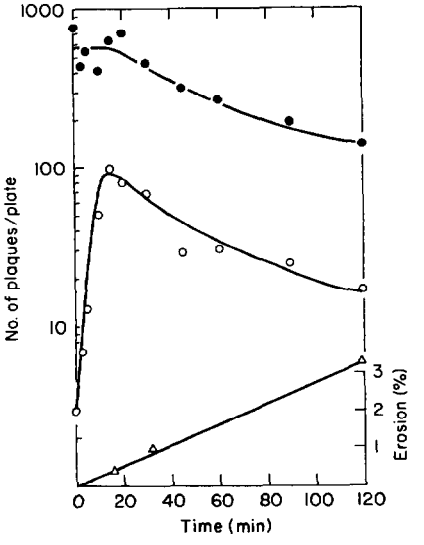

FIG. 10. Transfection of *E. coli* r⁻ and r⁻ *rec*B *rec*C *sbc*B strains as a function of time of erosion of P22 DNA.

Open and closed circles refer to *E. coli* r⁻ and r⁻ *rec*B *rec*C *sbc*B strain, respectively (left-hand scale). Triangles indicate level of erosion of P22 DNA with λ exonuclease (right-hand scale). Data in the Figure are not corrected for the 23% higher competence of the latter strain, measured with λ DNA as described in Materials and Methods.

than a restrictionless strain unimpaired in these functions (Fig. 10, zero-time samples). In addition, while λ exonuclease erosion of the terminally redundant regions of P22 DNA elicits an enhanced transfection in the latter strain, no such increase is obtained on the former (Fig. 10). Overoptimal erosion slowly decreases the transfecting activity of DNA in both cellular environments. These results indicate that intracellular exonucleolytic degradation is an important factor in lowering the transfecting efficiency of P22 DNA. Furthermore, they suggest that the activation achieved through *in vitro* erosion is due to a higher rate of escape of DNA molecules from the recBC nuclease mediated degradation (Benzinger *et al.*, 1975), and possibly also from attacks by the exonuclease I. The absence of these two functional exonucleases cancels the affects of erosion *in vitro*, and allows a 100-fold higher proportion of uneroded transfecting DNA molecules to enter pathways leading to the production of phage particles.

4. Discussion

P22 DNA molecules can be eroded with exonuclease III or λ exonuclease: both reactions show no lag as expected if both ends of the DNA molecules are completely base-paired. For most of the experiments reported here, λ exonuclease has been preferred to exonuclease III, because of its higher purity and inability to act at nicks (Cassuto *et al.*, 1971) which have been shown by alkaline sucrose sedimentation to be present in the proportion of about one out of five DNA molecules following erosion by λ exonuclease.

Molecules whose erosion approaches or exceeds 1% anneal extensively, forming unit size and larger circles as well as linear concameters. These data indicate that the redundant regions of *ts*$C_2$29 of P22 DNA comprise not more than 1% of the genome, less than 3%, as reported previously for the wild-type phage (Rhoades *et al.*, 1968) or 2% as found by Tye *et al.* (1974), for a C_I7 mutant phage. This discrepancy might be due to the fact that the phage used in this work is different from those studied earlier; indeed, Tye *et al.* (1974) have found that the lengths of the terminal repetitions of the DNA from different phage are not identical.

It is important to stress that the validity of our conclusion as for the length of the terminal repetitions rests on the assumption that the erosion of DNA molecules is synchronous. Our data argue that, if the asynchrony is defined as the difference in the levels of erosion between DNA molecules and expressed as a fraction of the genome, λ exonuclease action must have been synchronous within 1% since erosion above that level does not lead to formation of additional annealable molecules (Fig. 6), while better than 90% of the molecules are eroded (as measured by the electron microscopy visualization of "bushes", see Results). Nevertheless, both the transfecting activity and the annealability of P22 DNA increase in monotonous fashion over a relatively broad erosion interval (between 0 and 1%, Figs 1, 4, 6 and 10) as contrasted to the stepwise increase observed with T7 DNA (Ehrlich *et al.*, 1976). This finding might indicate asynchrony of erosion of P22 DNA within the 1% level; alternatively the length of terminal repetitions on different DNA molecules might not be identical. Such a possibility is not ruled out by the data of Tye *et al.* (1974): following a denaturation–renaturation cycle of C_I 7P22 DNA, they reported a direct electron microscopic measurement of the mean length of the single-stranded terminal repetitions to be 2·2% of the genome with a standard deviation of ±0·7.

The augmentation of biological activity of DNA molecules by enzymatic "degradation" is a novelty. The following is a plausible explanation. A multitude of obstacles must be averted by the transfecting P22 genome, if it is to fulfill its potential of giving rise to the phage progeny. Among those is the recBC nuclease of the host; the experiment reported in Figure 10 indicates that this enzyme, when functional, inactivates DNA molecules taken up by the cells at a frequency of 99 out of 100. Complete erosion of the redundant part of the phage genome enables it to form hydrogen-bonded circles (presumably gapped) which can then be converted by host enzymes into covalently closed molecules, known to be refractory toward the recBC enzyme (Karu *et al.*, 1973). A conversion of linear into circular DNA molecules was already observed in the normal infectious cycle of the P22 phage (Rhoades & Thomas, 1968). The efficiency of intracellular processing postulated above for *in vitro* exonuclease-treated DNA seems extraordinary: out of 99 molecules, which would normally be degraded, more than 20 survive when eroded (a 20-fold increase upon erosion, Figures 1 and 4, as compared to 100-fold higher transfectability of the *recB recC* host, Figure 10). When the recBC enzyme has been inactivated by a mutation, erosion should and does have no further beneficial effect (Fig. 10).

Erosion past the point of maximal activation brings forth a slow decrease of the transfecting activity both in cells of *rec*$^+$ and *rec*B *rec*C *sbc*B genotype. This is not due to the endonucleolytic degradation of DNA during the reaction; alkaline sucrose gradient analysis has shown the absence of nicks in λ exonuclease-eroded DNA.

Possible explanations are: (1) that the larger the single-stranded gaps in a circular DNA molecule, the more likely they are to be degraded by single-strand-specific nucleases as they (a) represent larger targets and (b) take longer to repair; (2) single-stranded termini might interfere with the uptake of transfecting DNA, the effect being commensurate with their length. The former hypothesis receives some support by the finding that in our system no transfecting activity is associated with denatured P22 DNA, while in the spheroplast system, which lacks periplasmic enzymes, denatured DNA is transfective (Lawhorne *et al.*, 1973). The latter hypothesis seems to us less likely given the wave-like variation of the transfecting activity with the increasing length of the single-stranded termini. Furthermore, unpublished results from this laboratory have shown that the addition of homopolymeric single-stranded sequences to the λ exonuclease-eroded DNA, catalyzed by the terminal transferase (Yoneda & Bollum, 1964), did not affect its transfectivity.

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